REMARKS

Status Summary

Claims 42-58 are pending. Claims 42, 43, 48, 49, 54-58 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing new matter. Claims 43, 47, 49, 51, and 53-58 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description of the claimed invention. Claims 48 and 54-58 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Claims 43, 45, 47, 48, 49, 51, and 53-58 are rejected under 35 U.S.C. § 102(e) as allegedly anticipated by U.S. Patent No. 5,993,816 to Lederman et al. Claims 42-58 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over U.S. Patent No. 5,993,816 to Lederman et al. in view of U.S. Patent No. 5,961,974 to Armitage et al. Claims 42-53, 54, 55, and 56-58 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over the above-noted references and further in view of U.S. Patent no. 4,937,183 to Ultee et al.

A Notice To Comply With Requirements for Patent Applications Containing Nucleotide Sequences and a Notice of Draftsperson's Drawing Review is included with the official action. The specification is objected to for informalities, organization, and use of non-preferred headings.

Claims 1-41 were canceled previously. Claims 43, 45, 47-56 are amended as indicated above. New claims 59-82 are added. A substitute specification, sequence listing, and formal drawings are submitted herewith. Reconsideration in view of the amendments and following remarks is respectfully requested.

Notice to Comply With Requirements
for Patent Applications Containing Nucleotide Sequences

A Notice To Comply With Requirements for Patent Applications Containing Nucleotide Sequences accompanied the official action mailed on January 8, 2003. Official Action, page 2, item 2. In accordance with 37 C.F.R. § 1.821(e), applicant requests to use the Sequence Listing filed in association U.S. Patent Application No. 08/338,975, filed November 14, 1994, now U.S. Patent No. 6,472,510, as stated in the letter submitted herewith. A paper copy of the Sequence Listing is also submitted herewith, which is believed to fully comply with the requirements of 37 C.F.R. §§ 1.821 through 1.825. Entry of the Sequence

Listing into the subject application is respectfully requested. The specification is amended to include sequence identifiers as set forth in the Sequence Listing.

Notice of Draftsperson's Drawing Review

A Notice of Draftsperson's Drawing Review accompanied the official action mailed January 8, 2003. Official Action, page 2, item 3. Formal drawings are submitted herewith, which are believed to fully comply with the requirements of 37 C.F.R. § 1.185. Acceptance of the drawings is respectfully requested.

Objections to the Specification

The examiner requests amendment of the specification (1) to update the status and relationship of the priority documents, (2) to adopt suggested section headings, (3) to delete the Table of Contents, (4) to properly reference trademarked material, and (5) to identify the current location of the American Type Culture Collection. Official Action, pages 3-4, items 4-6, page 7, item 10. The specification has been amended accordingly, and a substitute specification is submitted herewith. Applicant notes that support for incorporation of the priority documents is found in the transmittal submitted on filing the instant application. Withdrawal of the objections to the specification is respectfully requested.

Rejection of Claims Under 35 U.S.C. § 112, First Paragraph
Based on New Matter

Claims 42, 43, 48, 49, 54-58 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing new matter. Specifically, the examiner contends that "methods for inhibiting humoral immunity" and methods employing the "monoclonal antibody 5c8" are not sufficiently described in the application as originally filed. Official Action, pages 4-5, item 8. This rejection is respectfully traversed.

With respect to humoral immunity, the examiner states that "[h]umoral immunity can comprise various cell types and mediators associated with the immune response . . . [and that] [h]umoral immunity is not limited to B cell activation and immunoglobulin production." Official Action, pages 4-5, item 8, citing The Illustrated Dictionary of Immunology (Cruse & Lewis, CRC Press, Boca Raton, 1995). Applicant notes that the cited dictionary defines humoral immunity as "[i]mmunity attributable to specific immunoglobulin antibody and present in the blood plasma, lymph, other body fluids, or tissues. . . . Antibodies that are the

messengers of humoral immunity are derived from B cells." Applicant further submits that the definition makes clear that humoral immunity *requires* antibodies produced by B cell activation, as disclosed in the instant application, and *may also include* involvement of T cells, as suggested by the examiner. The instant specification discloses that the compositions of the invention are relevant to the treatment of humoral immunity disorders (*see e.g.*, pages 19-21). Thus, applicant believes that the term "humoral immunity" is adequately supported by the subject application as originally filed.

Based on the foregoing, claims 42, 43, 48, 49, 54-58 are believed to fully comply with the requirements of § 112, first paragraph, and withdrawal of the rejection of claims 42, 43, 48, 49, 54-58 is respectfully requested.

Rejection of Claims Under 35 U.S.C. § 112, First Paragraph

Based on Incomplete Description

Claims 43, 47, 49, 51, and 53-58 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description of the claimed invention. Specifically, the examiner contends that the term "CD40R" is insufficiently described because relevant identifying characteristics such as structure of other physical and/or chemical characteristics of CD40CR are not set forth in the specification commensurate in scope with the claims. The examiner also contends that there is insufficient written description of the genus of CD40CR proteins, including human CD40CR, and that applicant appears to rely on the limited disclosure of a mouse CD40CR. Official Action, pages 5-7, item 9. This rejection is respectfully traversed.

The Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement (herein after the "Guidelines") teach that "[t]o satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention." 66 Fed. Reg. 1099, 1104 (2001). Possession of the invention can be shown in any one of several ways, including by a description of physical properties in combination with a description of functional characteristics. *Id.* at 1106.

Applicant initially responds that each of claims 43, 45, 47, 49, 51, and 53 refers to a CD40CR protein in terms of its physical properties, *i.e.*, a 39 kD protein located on T helper

cell membranes. Support for the amendment is found in the application as originally filed, including at, for example, page 2 line 27 through page 3, line 4.

In addition to a description of CD40CR physical properties, each of claims 43, 45, 47, 49, 51, and 53 additionally describes functional properties of a CD40CR protein, including an ability to bind CD40 B-cell antigen and to stimulate B-cell cycle entry, proliferation, and differentiation. Techniques for determining the claimed functional properties are described in the application as originally filed, including, for example, at page 13, line 30, through page 15, line 36. Applicants respectfully submit that none of claims 43, 45, 47, 49, 51, and 53 refer to a CD40CR protein based solely on this functional description. Rather, in accordance with the Guidelines, a functional description is recited in addition to a description of the physical properties of a CD40CR protein.

Applicant further responds that the specification adequately describes the term "CD40CR," as used in the claims to encompass mammalian CD40CR proteins, which are specifically bound by antibodies of the present invention. The specification describes binding of monoclonal antibody MR1 to mouse CD40CR (Example 1) and to CD40CR-expressing human cells (Example 2). At the time of filing the instant application, CD40CR proteins had been described in several species, including human. *See e.g.*, Hollenbaugh et al., The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity, *EMBO J* 11 (12):4313-4321 (1992), and Spriggs et al., Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion, *J Exp Med* 176 (6):1543-1550 (1992). Thus, the art makes clear that a skilled artisan would understand the term "CD40CR," and following a review of the disclosure of the instant application, would conclude that the applicant was in possession of the necessary common attributes possessed by antibodies that bind to CD40CR.

Based on the foregoing arguments, the instant application is believed to fully describe the invention of claims 43, 45, 47, 49, 51, and 53 in accordance with the requirements of § 112, first paragraph. Claims 54-58 depend from claims 43, 45, 47, 49, 51, and 53, and are therefore also believed to be fully described. Thus, applicant respectfully requests withdrawal of the rejection of claims 43, 45, 47, 49, 51, 53, and 54-58.

Rejection of Claims Under 35 U.S.C. § 112, Second Paragraph

Claims 48 and 54-58 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite in recitation of "5c8," which does not correspond to the antibody having ATCC No. HB 11048. Claims 54-55 are further rejected under 35 U.S.C. § 112, second paragraph, as allegedly lacking antecedent basis for the term "fragment thereof." Claims 55-56 are still further rejected under 35 U.S.C. § 112, second paragraph, as allegedly broadening the claim scope to include an antibody conjugate. Official Action , page 8, item 11. These rejections are respectfully traversed.

Claim 48 is amended as directed to, *inter alia*, the MR1 antibody and binding fragments thereof. Claims 54-58 depend in part from claim 48 and thus are also directed to the MR1 antibody and MR1 fragments.

Claim 54 is amended to correct the perceived lack of antecedent basis by reciting functional properties of the antibody fragments, as suggested by the examiner. Claim 55 is amended to delete the language "or fragment thereof."

Claims 55-56 are amended to correct the perceived lack of antecedent basis by inclusion of the language "further comprising" in reference to a moiety (claim 55) or a therapeutic agent (claims 56), wherein the moiety or therapeutic agent is linked to the antibody. Support for the amendments can be found in the application as originally filed, including at page 11, lines 25-35, which describe linking the disclosed antibodies to a second molecule. In addition, as noted by the examiner, antibody conjugates are well known in the art. Official Action, page 10, item 16.

Based on the foregoing arguments, claims 48 and 54-58 are believed to fully comply with the requirements of 35 U.S.C. § 112, second paragraph, and withdrawal of the rejections under § 112, second paragraph, is respectfully requested.

Rejection of Claims Under 35 U.S.C. §§ 102(e) and 103(a)

Claims 43, 45, 47, 48, 49, 51, and 53-58 are rejected under 35 U.S.C. § 102(e) as allegedly anticipated by U.S. Patent No. 5,993,816 to Lederman et al. (Lederman). The '816 patent describes methods for inhibiting humoral immune responses via administration of a 5c8 antibody, which binds to human CD40CR. Official Action, page 9, item 14. Claims 42-58 are rejected under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,993,816 to Lederman et al. (Lederman) in view of U.S. Patent No. 5,961,974 to Armitage et

al., and further in view of U.S. Patent No. 4,937,183 to Ultee et al. Official Action, pages 9-10, item 15. The rejections based on Lederman are respectfully traversed based on applicant's request for interference under 37 C.F.R. §§ 1.607 and 1.608(a), which was submitted via a preliminary amendment filed on December 20, 1999, and which is summarized herein below. Elimination of Lederman as prior art renders moot the rejections under §§ 102(e) and 103(a).

Discussion of New Claims

New claims 59-82 are added to describe additional embodiments of the invention. Specifically, new claims 59-64 are directed to methods employing monoclonal antibody MR1 produced by the hybridoma having ATCC Accession No. HB 11048, and fragments thereof having the same binding specificity; new claims 65-70 are directed to methods employing a human antibody comprising an MR1 binding fragment; new claims 71-76 are directed to methods employing a chimeric antibody comprising an MR1 binding fragment; and new claims 77-82 are directed to methods employing an F(ab')2 fragment of the MR1 antibody. Support for new claims 59-82 is found in the application as originally filed, including, for example, at page 31, wherein reference to ATCC Accession No. HB 11048 is made; at page 11, lines 25-28, wherein antibody molecules comprising an antigen combining site that binds to CD40R are described; at page 12, lines 1-14, wherein methods for producing human antibodies are described; at page 12, lines 27-32, wherein methods for producing chimeric antibodies are described; and at page 12, lines 15-26, wherein methods for producing $F(ab')_2$ fragments are described. Entry of new claims 59-82 is respectfully requested.

Request for Interference Pursuant to 37 C.F.R. §§ 1.607(a) and 1.608(a)

Further to applicant's request for interference filed with a preliminary amendment and with the instant application on December 20, 1999, applicant reiterates that a *prima facie* showing to support declaration of interference in accordance with 37 C.F.R. §§ 1.607(a) and 1.608(a) has been made. Applicant further submits that the examiner has failed to notify applicant of the reasons for denial of the interference and has failed to expedite examination as required by 37 C.F.R. § 1.608(b).

I. Applicant Has Made *Prima Facie* Showing to Support Declaration of Interference

Previously added claims 42-58 and new claims 59-82 are directed to methods of using antibodies specific to CD40L (also known in the art as CD40CR, gp39, CD154, 5c8 antigen, TBAM) to inhibit humoral immunity, immunoglobulin production and B-cell activation. Inhibition results upon antibody binding to CD40L, which is an antigen expressed on activated T-cells that is involved in contact-dependent T-cell activation of B-cells. The subject matter of the instant claims closely parallels that of claims issued in U.S. Patent No. 5,993,816 to Lederman et al.

Rule 607(a) provides that an applicant may seek to have an interference declared with a patent by (1) identifying the patent, (2) presenting a proposed count, (3) identifying at least one claim in the patent corresponding to the proposed count, (4) presenting at least one claim corresponding to the proposed count, (5) showing support for newly presented claims in the disclosure of the application, and (6) explaining how the requirements of 35 U.S.C 135(b) are met, if the claim(s) presented were not present in the application until more than one year after the issue date of the patent. 37 C.F.R. § 1.607(a).

In accordance with Rule 607(a), applicant's preliminary amendment filed on December 20, 1999, (1) identified U.S. Patent No. 5,993,816 to Lederman et al. as the patent with which interference is sought (page 9); (2) proposed a count, (page 12); (3) identified claims 1-14 of the Lederman '816 patent as corresponding to the count (pages 13-16); (4) presented new claims 42-58 as corresponding to the count (pages 2-6, 17); (5) identified the basis for new claims 42-58 in the application as originally filed (pages 7-8); and (6) stated that the requirements of 35 U.S.C. § 135(b) are met based on presentation of claims less than one year after issuance of the '816 patent (page 16). A copy of the preliminary amendment is submitted herewith for the examiner's convenience.

When the effective filing date of an application is less than three (3) months after the effective filing date of a patent with which an interference is sought, as in the present case, Rule 608(a) provides that a *prima facie* showing by the applicant is "a statement alleging that there is a basis upon which the applicant is entitled to a judgment relative to the patentee." 37 C.F.R. § 1.608(a). Applicant respectfully submits that such a statement has been made with respect to previously added claims 42-58. Amendments to claims 43, 45, 47, and 48-58 are believed to place the claims in condition for allowance and do not alter claim

correspondence to the proposed count. New claims 59-82 are directed to substantially the same subject matter as claims 42-58, *i.e.* methods of using antibodies specific to CD40L to inhibit humoral immunity, immunoglobulin production and/or B-cell activation, and thus also correspond to the proposed count.

Based on the foregoing, applicant respectfully requests prompt declaration of interference between the instant application and U.S. Patent No. 5,993,816.

II. The Examiner has Failed to Notify Applicant of Reasons for Denial of Request for Declaration of Interference

Rule 607(b) provides that "[i]f the examiner determines that there is no interfering subject matter, the examiner shall state the reasons why an interference is not being declared and otherwise act on the application." 37 C.F.R. § 1.607(b).

In the instant case, the examiner issued an official communication mailed March 26, 2001, notifying applicant that all claims were allowable and that prosecution was suspended due to a potential interference. A similar official communication was mailed on November 30, 2001. The examiner then issued an official action mailed January 8, 2003, to which applicant now responds. The official action did not include, however, a statement as required under Rule 607(b). In particular, the examiner did not clarify why he twice previously found all claims allowable, and later retracted this position by issuing a rejection of claims.

III. The Examiner has Failed to Expedite Prosecution

Rule 607(b) also provides that when declaration of interference is requested by an applicant "examination of the application, including any appeal to the Board, shall be conducted with special dispatch within the Patent and Trademark Office." 37 C.F.R. § 1.607(b).

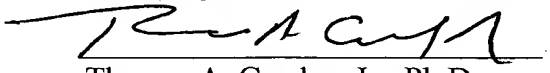
Despite the requirements of Rule 607(b), there has been significant delay in responding to the present request for interference. Specifically, the examiner stated in an interview on September 18, 2000, "will set up interference in near future, hopefully by end of October." As noted herein above, the examiner stated in official communications mailed March 26, 2001, and November 30, 2001, that all claims were allowable and that prosecution was suspended due to a potential interference. An official action on the merits was mailed on January 8, 2003, more than three years after filing of the application. Applicant respectfully submits that this delay was in violation of Rule 607(b).

Conclusion

The rejections under 35 U.S.C. § 112 having been addressed, it is respectfully submitted that the present application is in condition for allowance save the examiner's rejection of claims under 35 U.S.C. § 102(e) and § 103(a) based on Lederman. In view of applicant's request for interference pursuant to 37 C.F.R. §§ 1.607 and 1.608(a), it is requested that an interference with U.S. Patent No. 5,993,816 now be declared. If any points remain in issue, which the examiner feels may be best resolved through a personal or telephone interview, he is kindly requested to contact the undersigned attorney at the telephone number listed below.

Respectfully submitted,

PILLSBURY WINTHROP LLP



Thomas A. Cawley, Jr., Ph.D.
Registration No. 40,944

P.O. Box 10500
McLean, VA 22102
(703) 905-2144 Direct Dial
(703) 905-2500 Facsimile

Date: June 9, 2003

TAC/JB

#13. Goiga

APPLICATION UNDER UNITED STATES PATENT LAWS

Atty. Dkt. No. PW 037003-0276453
(M#)

Invention: METHOD FOR PROLONGED SUPPRESSION OF HUMORAL IMMUNE RESPONSE TO A THYMUS-DEPENDENT ANTIGEN THERAPEUTIC AGENT

Inventor (s): Randolph NOELLE

For correspondence Address

00909
Pillsbury Winthrop LLP

This is a:

- ☐ Provisional Application
- ☐ Regular Utility Application
- ☐ Continuing Application
  - ☒ The contents of the parent are incorporated by reference
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
  Sub. Spec Filed _____
  in App. No. _____
- ☒ Marked up Specification re
  Sub. Spec. filed June 9, 2003
  In App. No 09/467,317

SPECIFICATION

USE OF ANTIBODIES THAT SPECIFICALLY BIND CD40R (cd40 LIGAND) TO INHIBIT HUMORAL IMMUNITY

RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application No. 08/742,480, filed November 1, 1996, which is ~~in turn~~ a continuation of U.S. Patent Application No. 08/338,975, filed November 14, 1994, now U.S. Patent No. 6,472,510, which is ~~in turn~~ a continuation of U.S. Patent Application No. U.S. 07/835,799, filed February 14, 1992, now abandoned, each of the foregoing applications incorporated herein in its entirety.

~~1.~~ INTRODUCTION FIELD OF THE INVENTION

The present invention relates to a counter-receptor, termed CD40CR, for the CD40 B-cell antigen, and to soluble ligands for this receptor, including fusion molecules comprising at least a portion of CD40 protein. It is based, at least in part, on the discovery that a soluble CD40/immunoglobulin fusion protein was able to inhibit helper T-cell mediated B-cell activation by binding to a novel 39 kD protein receptor on helper T-cell membranes. The present invention provides for a substantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion molecules comprising at least a portion of CD40 protein; and for methods of controlling B-cell activation which may be especially useful in the treatment of allergy or autoimmune disease.

~~2.~~ BACKGROUND OF THE INVENTION

Studies by Mitchison, Benacerraf and Raff first suggested that physical interactions between T_h and B-cells were essential in the development of humoral immune responses. Later studies documented that T_h formed physical conjugates with class II major histocompatibility complex (MHC) compatible, antigen-presenting B-cells (*Vitetta et al., (1987) Immunol. Rev. 99:193-239)* and that it was the B-cells within these conjugates that responded to T_h (*Bartlett et al., (1989) J. Immunol. 143:1745-1754).*

With the discovery that T_h-derived lymphokines exerted potent growth and differentiative effects on B-cells, it was proposed that soluble factor(s) released in proximity by activated T_h mediated the activation of the interacting B-cell. However, none of the molecularly cloned lymphokines, alone or in combination, manifested the ability to induce B-cell cycle entry. Unlike soluble factors, plasma membrane fractions from activated T_h induced B-cell cycle entry *(Hodgkin et al., (1990) J. Immunol. 145:2025-2034; Noelle et al., (1991) J. Immunol. 146:1118-1124)*. Studies using purified plasma membrane fractions from activated T_h suggested that a protein expressed on the membrane of activated T_h was responsible for initiating humoral immunity (*Noelle et al., (1991) J. Immunol. 146:1118-1124; Bartlett et al., (1990) J. Immunol. 145:3956-3962).*

Purified plasma membranes from activated T_h (PM^{Act}) have been used to investigate the nature of this effector function *(Hodgkin et al., (1990) J. Immunol. 145:2025-2034: Noelle et al., (1991) J. Immunol. 146:1118-1124).* PM^{Act} from activated T_h, but not resting T_h (PM^{rest}) expressed on activity that induced B-cell cycle entry in an antigen-nonspecific, class II-unrestricted manner. In addition, it was shown that the activity expressed by PM^{Act} required 4-6 hours of activation, de novo RNA synthesis and was protein in nature (*Bartlett et al., (1990) J. Immunol. 145:3956-3962).*

SUMMARY OF THE INVENTION

The present invention relates to a counter-receptor, termed CD40CR, for the CD40 B-cell antigen, and to soluble ligands for the receptor, including fusion molecules comprising at least a portion of CD40 protein. It is based, at least in part, on the discovery that a soluble CD40/immunoglobulin fusion protein was able to inhibit helper T-cell mediated B-cell activation by binding to a novel 39 kD receptor protein (termed "CD40CR" for CD40 counter-receptor) on helper T-cell membranes, and on the

discovery that a monoclonal antibody, termed MR1, directed toward this 39 kD receptor was able to inhibit helper T-cell mediated activation of B-cells.

The present invention provides for a substantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion molecules comprising at least a portion of CD40 protein; and for methods of controlling B-cell activation.

In particular embodiments of the invention, B-cell activation in a subject may be inhibited by contacting helper T cells of the subject with effective amounts of a soluble ligand of CD40CR. Such inhibition of B-cell activation may be especially useful in the treatment of the allergy or autoimmune disease.

One advantage of the present invention is that it enables intervention in an aspect of the immune response which is not antigen specific. Many current therapies for allergy include desensitization to particular antigens, and require that each patient be tested in order to identify antigens associated with sensitivity. As a practical matter, exhaustive analysis of a patient's response to each and every potential allergen is virtually impossible. Furthermore, in most autoimmune conditions, the causative antigen is, generally, unknown or even irrelevant to the disease process. The present invention, which relates to the antigen nonspecific CD40/CD40CR interaction, circumvents the need to characterize the antigen associated with allergy or autoimmunity. Therefore, the present invention may be used to particular advantage in the treatment of allergic conditions in which the immunogen is not known, or has multiple components, for example, in hay fever or in procainamide induced lupus. It may also be useful in acute treatment of immune activation, for example, in therapy for anaphylaxis.

~~3.1~~ ABBREVIATIONS

Ig immunoglobulin

mab	monoclonal antibody
PM^{Act}	plasma membranes prepared from activated helper T-cells
PM^{rest}	plasma membranes prepared from resting helper T-cells
PAGE	polyacrylamide gel electrophoresis
rIL4	recombinant interleukin 4
rIL5	recombinant interleukin 5
SN	supernatant
T_h	helper T-cell
T_h1	refers to D 1.6, a I-A^d-restricted, rabbit immunoglobulin specific clone

~~4. DESCRIPTION OF THE FIGURES~~

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of monoclonal antibodies and CD40-Ig on the induction of B-cell RNA synthesis by PM^{Act}.

Panel A. Resting B-cells were cultured with PM^{rest} or PM^{Act} from T_h1. 25μg/ml of anti-CD4, anti-LFA-1 or anti-ICAM-1 or a combination of each of these (each at 25 μg/ml) was added to wells containing $PM^{Act,}$ and B cell RNA synthesis was measured by incorporation of [^{3}H]-uridine. B-cell RNA synthesis was assessed from 42 to 48 hours post-culture. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 5 such experiments.

Panel B. Resting B-cells were cultured with PM^{Act} from T_h1 (●,▲) or T_h2 (□). To the T_h1 PM^{Act} containing culture (●,▲), increasing amounts of CD40-Ig (▲) or control protein CD7E-Ig (●) were added To the T_h2 PM^{Act} containing culture (□), increasing amounts of CD40-Ig were added. B-cell RNA synthesis was assessed from 42 to 48 hours post-culture. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Panel C. Resting B-cells were cultured with LPS (50μg/ml) or PM^{Act}. To cultures, CD40-Ig (25 μg/ml; hatched) or CD7E-Ig (25 μg/ml; solid) were added. RNA synthesis was determined as described in Panel A. Results presented are the arithmetic mean of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Figure 2. CD40-Ig inhibited B-cell differentiation and proliferation.

Panel A. Resting B-cells were cultured with PM^{Act}, rIL4 (10 ng/ml) and rIL5 (5ng/ml). Either at the initiation of culture, or on days 1, 2 or 3 post-initiation of culture, CD40-Ig or CD7E-Ig (25μg/ml) were added. On day six of culture, SN from individual wells were harvested and quantitated for IgM (■) and IgG_1 (●) using an anti-isotype specific ELISA, as described in *(Noelle et al., (1991) J. Immunol. 146:1118-1124).* In the presence of PM^{Act}, IL4 and IL5, (in the absence of added CD40-Ig) the concentrations of IgM and IgG_1 were 4.6 μg/ml and 126 ng/ml, respectively. Cultures which received CD7E-Ig (25 μg/ml) on Day 0 produced 2.4 μg/ml and 89 ng/ml of IgM and IgG_1 respectively. In the absence of IL4 and IL5, no IgM or IgG_1 was detected. Results are representative of 3 such experiments.

Panel B. T_h1 were rested or activated with anti-CD3 for 16 hours, irradiated and cultured ($1x10^4$/well) with resting B-cells ($4X10^4$/culture) in the presence of IL4 (10 ng/ml). Between 0 and 25 μg/ml of CD40-Ig (▲) or CD7E-Ig (●) were added to cultures. From 66-72 hours post-culture, wells were pulsed with 1.0 μCi of [^{3}H]-thymidine and harvested. The dotted line indicates the response of B-cells to resting T_h. Results presented are the arithmetic mean of triplicate cultures +/- s.d., and are representative of 2 such experiments.

Figure 3. CD40-Ig detected a molecule expressed on activated, but not resting T_h. Resting and activated, T_h were harvested and incubated with fusion proteins for 20

minutes at 4°C, followed by FITC-conjugated goat anti-hIgG (25 μg/ml). Percentage positive cells and MFI were determined by analysis of at least 5000 cells/sample. Results are representative of 6 such experiments. CD40-Ig binding is indicated by a filled-in profile.

Figure 4. CD40-Ig immunoprecipitated a 39 kD protein from lysate of activated T_h1. T_h1 were rested or activated with insolubilized anti-CD3 for 16 hours. [^{35}S]-labelled proteins from resting or activated T_h were immunoprecipitated with purified antibodies or fusion proteins (1-10μ). The gel profile is representative of 3 such experiments.

Figure 5. A monoclonal antibody (mab), specific to the induced 39 kD T_h membrane protein, inhibited induction of B-cell RNA synthesis by PM^{Act}. Resting B-cells and PM^{Act} were cultured with 10 μg/ml each of anti-α/β, anti-CD3, CD40-Ig or MR1. RNA synthesis was determined as described in Figure 1. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Figure 6. MR1 and CD40-Ig recognized the same molecule expressed on activated T_h.

Panel A: Activated T_h were fluorescently stained with MR1 or control Ig. To evaluate if CD40-Ig and MR1 competed for binding to activated T_h, graded concentrations of MR1 or control hamster Ig (anti-α/β TCR) were added together with anti-CD40 (20μg/ml). After incubation for 20 minutes at 4°C, the samples were washed and incubated with FITC-conjugated, mab anti-human IgG_1. Results are representative of 3 such experiments.

Panel B: Proteins from [^{35}S]-methionine-labelled, activated T_h were immunoprecipitated with MR1 (10 μg/sample) or CD40-Ig (10 μg/sample) and resolved by PAGE and fluorography. Results presented are representative of 2 such experiments.

Figure 7. Binding of CD40-Ig to human cell lines. A variety of human T-cell lines were exposed to biotin-labelled CD40-Ig, and binding was evaluated by flow cytometry.

Figure 8.

Panel A: Nucleotide sequence of CD40 cDNA from *Stamenkovic et al., (1989) EMBO J. 8:1403-1410.* The transmembrane region is underscored.

Panel B: Schematic diagram of a plasmid that may be used to express CD40-Ig. The amino acid sequences at the site of fusion of Δ CD40 is shown below the diagrammed portion of CD40 (SEQ ID NO:3).

~~5.~~ DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for Δ substantially purified CD40CR receptor; for soluble ligands of CD40CR, including antibodies as well as fusion molecules comprising CD40; and for methods of controlling B-cell activation.

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) ligands that bind to CD40CR;

(ii) methods used to characterize CD40CR;

(iii) preparation of purified CD40CR;

(iv) uses of ligands that bind to CD40CR;

and

(v) uses of CD40CR.

~~5.1 LIGANDS THAT BIND TO CD40CR~~

Ligands that Bind to CD40CR

The present invention provides for soluble ligands of CD40CR, including (i) fusion molecules comprising at least a portion of CD40 protein and (ii) antibodies or antibody fragments.

The term "soluble", as used herein, indicates that the ligands of the invention are not permanently associated with a cell plasma membrane. Soluble ligands of the invention may, however, be affixed to a non-cellular solid support, including a lipid, protein, or carbohydrate molecule, a bead, a vesicle, a magnetic particle, a fiber, etc. or may be enclosed within an implant or vesicle.

The ability of such a ligand to bind to CD40CR may be confirmed by demonstrating that the ligand binds to the same protein as CD40-Ig (infra) or MRI (infra).

The ligands of the invention may be comprised in pharmaceutical compositions together with a suitable carrier.

~~5.1.1 FUSION MOLECULES~~

Fusion Molecules

The present invention provides for soluble fusion molecules that are ligands of CD40CR. Such fusion molecules comprise at least a portion of CD40 protein attached to a second molecule. The portion of CD40 preferably lacks the CD40 transmembrane domain. A portion of CD40 protein which may be used according to the invention is

defined as any portion which is able to bind to CD40CR, for example, such a portion may be shown to bind to the same protein as MR1 or CD40-Ig.

Second molecules which may be used include peptides and proteins, lipids, and carbohydrates, and, in preferred embodiments of the invention, may be an immunoglobulin molecule, or portion thereof (such as an Fv, Fab, $F(ab')_2$, for Fab' fragment) or CD8, or antoher adhesion molecule, such as B7. The second molecule may be derived from either an non-human or a human source, or may be chimeric. The second molecule may also be an enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The fusion molecules of the invention may be produced by chemical synthesis or, preferably, by recombinant DNA techniques.

For example, a nucleic acid sequences encoding at least a portion of CD40 protein may be combined with a nucleic acid sequence encoding a second molecule in a suitable expression vector, and then expressed in a prokaryotic or, preferably, eukaryotic expression system, such as a yeast, baculovirus, or mammalian expression system, including transgenic animals.

Alternatively, at least a portion of CD40 protein may be expressed using recombinant DNA techniques and then may be chemically conjugated to a second molecule.

Fusion molecules comprising CD40 may be purified from preparative mixtures using electrophretic techniques or affinity chromatography using ligand that binds to either CD40 or to the second molecule. Ligands that bind to CD40 include, but are not

limited to, anti-CD40 antibodies such as G28-5, as produced by the hybridoma having accession number HB9110 and deposited with the American Type Culture Collection, and CD40CR, described more fully in sections 5.2 and 5.3, infra. If the second molecule is an immunoglobulin or immunoglobulin fragment, an affinity column comprising anti-immunoglobulin antibody may be used; if the second molecule comprises an F_c fragment, a protein A column may be used.

According to a preferred embodiment of the invention, a portion of CD40 may be produced using a nucleic acid sequence that encodes a CD40 protein that is truncated upstream from the transmembrane domain. Such a nucleic acid sequence may be prepared by digesting a plasmid containing cDNA encoding CD40 antigen, such as that described in *Stamenkovic et al., (1989), EMBO J. 8:1403-1410*, with PstI (P) and Sau 3A (S3) restriction enzymes. The resulting P/S3 fragment may be subcloned into the same plasmid digested with P and Bam HI (B), to produce a truncated CD40 gene (see Figure 8).

In particular, nonlimiting, embodiments of the invention, an expression vector used to produce ligands containing at least a portion of CD40 as well as immunoglobulin sequence may preferably comprise a virally-derived origin of replication, a bacterial origin of replication, a bacterial selectable marker, and eukaryotic promoter and enhancer sequences separated from DNA sequences encoding an immunoglobulin constant region by restriction endonuclease sites which allow subcloning of DNA sequences encoding at least a portion of CD40, followed by a polyadenylation signal sequence (see Figure 8.b.).

In a specific embodiment of the invention, the truncated CD40 gene may be subcloned into an immunoglobulin fusion plasmid, such as that described in *Aruffo et al., 1990, Cell 61:1303-1313,* using an Mlu I and B digest, to form plasmid pCD40-Ig, which

encodes the fusion molecule CD40-Ig (see Figure 8). CD40-Ig fusion protein may then be produced by transfecting the pCD40-Ig plasmid into COS cells to form a transient expression system. CD40-Ig produced may be collected from the COS cell supernatant and purified by protein A column chromatography as described in *Aruffo et al., 1990, Cell 161:1303-1313.*

~~5.1.2. ANTIBODIES~~

Antibodies

The soluble ligands of the invention may comprise antibody molecules, monoclonal antibody molecules, or fragments of these antibody molecules which contain an antigen combining site that binds to CD40CR. Such ligands may further comprise a second molecule which may be protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound and may be linked to the antibody molecule or fragment.

Where the ligand is a monoclonal antibody, or a fragment thereof, the monoclonal antibody can be prepared against CD40CR using any technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497) as well as other techniques which have more recently become available, such as the human B-cell hybridoma technique (Kozbar et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96) and the like are within the scope of the present invention.

Antibody fragments which contain the idiotype of the molecule could be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be generated by treating the antibody molecule with pepsin; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment; the F(ab')2 fragment which can be generated by treating the antibody molecule with papain; and the 2Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent to reduce the disulfide bridges.

The present invention also provides for chimeric antibodies produced by techniques known in the art, such as those set forth in *Morrison et al., (1984) Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855 or European Patent Application No. 85305604.2, publication No. 0173494 by Morrison et al., published March 5, 1986.*

Immunogen for the production of antibodies may be any source that contains CD40CR. For example, activated T_h may be used as an immunogen.

Alternatively, substantially purified CD40CR, prepared as set forth in section 5.3, infra, may be used. If activated T_h are used as immunogen, antiserum may be tested for reactivity against activated but not resting T_h cells.

In a preferred embodiment of the invention, the soluble ligand is the MR1 monoclonal antibody. The following method was used to produce the MR1 monoclonal antibody, and may be used to generate other antibodies directed toward CD40CR.

Hamsters were immunized intraperitoneally with $5\text{-}10^6$ activated T_h1 cells (D1.6) at weekly intervals for six weeks. When the serum titer against murine T_h1 was greater than about 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NSI. SN from wells containing growing hybridomas were

screened by flow cytometry on resting and activated T_h1. One particular hybridoma, which produced a mab that selectively recognized activated T_h, was further tested and sublconed to derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC.

The present invention also provides for ligands comprising monoclonal antibodies, and fragments thereof that are capable of competitively inhibiting the binding of MR1 to its target antigen or CD40-Ig to its receptor.

~~5.2 METHODS USED TO CHARACTERIZE CD40CR~~

Methods Used to Characterize CD40R

CD40CR may be characterized by (i) its ability to bind CD40, fusion molecules comprising at least a portion of CD40, and antibodies such as MR1; (ii) its functional characteristic of being able to stimulate B-cell cycle entry, proliferation, and differentiation; and (iii) its cellular distribution.

~~5.2.1 ABILITY TO BIND LIGANDS~~

CD40CR may be characterized by its ability to bind to ligands such as CD40, fusion molecules comprising CD40, and antibodies directed toward CD40CR.

As discussed in greater detail infra, several techniques were used to characterize CD40CR. For example, CD40-Ig and MR1 were shown to recognize the same 39 kD molecule. Both CD40-Ig and MR1 were found to immunoprecipitate a 39 kD protein from radiolabelled T_h lysates (Figure 5b). Further, immunoprecipitation of the 39 kD protein with CD40-Ig removed the antigen recognized by MR1 from T_h lysates.

~~5.2.2. ABILITY TO STIMULATE B-CELLS~~

CD40CR may also be characterized by its ability to stimulate B-cell cycle entry, proliferation, and differentiation.

For example, plasma membrane (PM) from activated (PM^{Act}) but not resting (PM^{rest}) T_h cells was found to induce B-cell RNA synthesis (Figure 1a); this induction, indicative of B-cell activation, was not; affected by antibodies such as anti-LFA-1, anti-CD4, anti-ICAM-1. CD40-Ig or MR1, however, were found to be able to inhibit PM^{Act} – induced B-cell activation, as shown in Figure 1b and Figure 6.

The induction of B-cell activation may be measured by techniques such as [^{3}H]-uridine incorporation into RNA (as B-cells differentiate, RNA synthesis increases), or by [^{3}H]-thymidine incorporation, which measures DNA synthesis associated with cell proliferation. For optimal measurement of the effect of CD40CR on B-cell proliferation, interleukin-4 (IL-4) may be added to the culture medium at a concentration of about 10 ng/ml.

Alternatively, B-cell activation may be measured as a function of immunoglobulin secretion. For example, CD40Cr, in substantially purified form, or as present in PM, or otherwise, may be added to resting B-cells together with IL-4 (10 ng/ml) and IL-5 (5 ng/ml). After three days of culture, an additional volume of culture medium may be added. On a day 6 of culture, supernatant (SN) from individual cultures may be harvested and quantitated for IgM and IG_1 as described in *Noelle et al., (1991) J. Immunol.* *146:1118-1124.*

~~5.2.3 CELLULAR DISTRIBUTION~~

CD40CR may also be characterized by its cellular distribution. For example, CD40-Ig was observed to bind to activated, but not resting T_h1, as assessed by flow

cytometry (Figure 3). Furthermore, CD40-Ig was observed to bind to Jurkat cells, HSB2 cells, and activated T-cells from human peripheral blood, but did not appear to bind significantly to CEM cells, HPBALL cells, or murine thymoma cells.

For example, and not by way of limitation, the presence of CD40CR on a particular cell type ("test cells") may be evaluated by flow cytometry as follows. Test cells may be tested in parallel with resting (negative control) and activated (positive control) T_h cells. All cells may be incubated at a concentration of about 1 x 10^5 cells/50 μl with ligand (e.g. CD40-Ig or MR1) for 20 minutes at 4°C, followed by FITC-conjugated anti-ligand antibody. Propidium iodide may be added to all samples to a final concentration of 2 μg/ml. Flow cytometric analysis may then be performed, for example on a BD FACSCAN. After positive gating of cells by forward versus side scatter, and by red negativity (for propidium iodide exclusion), and the log green fluorescence of viable cells may be ascertained.

~~5.3 PREPARATION OF PURIFIED CD40CR~~

Preparation of Purified CD40CR

The present invention provides for substantially purified CD40CR. Such CD40CR may be prepared from cells bearing CD40CR, such as activated helper T-cells, Jurkat, and HSB2 cells, by the following method.

Plasma membranes may be prepared from appropriate cells, such as activated T_h1 cells, by discontinuous sucrose gradient sedimentation, as described in *Noelle et al., 1991, J. Immunol. 146:1118-1124.* CD40CR may then be isolated by dissociating the crude membrane extract with mild detergent, and then performing size exclusion chromatography followed by either affinity chromatography using appropriate ligands

(e.g., MR1 or CD40-IG) bound to a solid support, immunoprecipitation (e.g. by CD40-IG or MR1), and/or gel electrophoresis.

The resulting protein may be expected to have a molecular weight of about 39 kD,

The present invention provides for a soluble CD40CR (i.e., cell-free) which may be comprised in pharmaceutical compositions together with a suitable carrier. It further provides for CD40CR which is linked to a second molecule which may be a peptide, protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The present invention further provides for substantially purified CD40CR which has been prepared by chemical synthesis or recombinant DNA techniques. For example, the gene for CD40CR may be isolated by inserting cDNA prepared from activated helper T-cells into the λgt10 expression system, and then screening with MR1 or CD40-Ig binding to identify CD40CR-expressing clones. Alternatively, cDNA prepared from activated helper T-cells may be transfected into COS cells, the supernatants of which may be screened with MR1 or CD40-Ig to identify CD40CR producers. The gene for CD40CR may be then used to express CD40CR using expression systems in the art.

~~5.4 USES OF LIGANDS THAT BIND TO CD40CR~~

Uses of Ligands that Bind to CD40CR

The present invention provides for methods of controlling B-cell activation that utilize ligands that bind to CD40CR. In particular, it provides for a method of inhibiting B-cell activation comprising exposing a mixture of B-cells and T_h cells to an effective concentration of ligand that binds to CD40CR. Ligands that may be used are described

supra in section 5.1. The method of the invention may be practiced in vitro or in vivo. An effective concentration refers to a concentration of a ligand that inhibits B-cell activation, measured by any technique known in the art (including those set forth in section 5.2, supra) by at least about 30 percent, and preferably by about 75 percent. According to a preferred, specific, non-limiting embodiment of the invention, CD40-Ig may be used as ligand, in which invention, CD40-Ig may be used as ligand, in which case an effective concentration may be at least about 10 μg/ml. In another specific, nonlimiting embodiment of the invention, the monoclonal antibody MR1 may be used, in which case an effective concentration may be at least about 10 μg/ml. If the method is practiced in vivo, an effective concentration of ligand may refer to plasma concentration of ligand or to a local concentration. For example, it may be desirable to inhibit B-cell activation in a localized area in order to limit the effects on the immune system as a whole.

In particular embodiments, the invention provides for a method of treating a subject suffering from a disorder associated with B-cell activation, comprising administering to the subject a therapeutic amount of ligand that binds to CD40CR. A subject may be a non-human or, preferably, a human animal.

Disorders associated with B-cell activation include, but are not limited to, allergy (including anaphylaxis); autoimmune conditions including drug induced lupus, systemic lupus erythematosus, adult rheumatoid arthritis, juvenile rheumatoid arthritis, scleroderma, Sjogren's Syndrome, et c.; and viral diseases that involve B-cells, including Epstein-Barr infection, and retroviral infection including infection with a human immunodeficiency virus.

Because it has been suggested that B-cell activation is associated with the induction of human immunodeficiency virus replication from latency, it may be desirable to administer the ligands of the invention to HIV positive individuals who have not yet developed AIDS or ARC.

Ligands may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular or intramuscular injection, and oral, intranasal, intraocular and rectal administration, and may be comprised in microspheres, liposomes, and/or sustained released implants.

A therapeutic amount of ligand is defined as an amount which significantly diminishes the deleterious clinical effects of B-cell activation, and may vary among ligands used and conditions treated. If CD40-Ig is used, therapeutic concentration may be about 10 μg/ml either systemically (plasma concentration) or locally. If MR1 is used, a therapeutic concentration may be about 10 μg/ml either systemically (plasma concentration) or locally.

In a further embodiment of the invention, the above methods may utilize a ligand comprising a toxin or antimetabolite such that T_h cells are killed or damaged and B-cell activation is decreased as a result of T_h cell destruction.

The ligands of the invention may also be used to label activated T cells, a technique which may be useful in the diagnosis of T cell disorders. To this end, ligand comprising an enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to T cells in vitro or in vivo and the amount of binding may be quantitated.

The ligands of the invention may also be used to deliver substances, e.g. growth factors, to be activated T-cells.

~~5.5 USES OF CD40CR~~

Uses of CD40CR

The present invention provides for methods of controlling B-cell activation that utilize CD40CR or a molecule comprising CD40CR, prepared as described in section 5.3, supra. In particular, it provides for a method of promoting B-cell activation comprising exposing B-cells to an effective concentration of CD40CR. The method may be practiced in vivo or in vitro. An effective concentration refers to a concentration of receptor that induces B-cell activation, measured by any technique known in the art (including those set forth in section 5.3, supra) by at least about 30 percent. In specific, nonlimiting embodiments of the invention, the concentration of CD40CR may be about to μg/ml locally or systemically.

In particular embodiments, the invention provides for a method of treating a subject suffering from an immunodeficiency disorder associated with diminished humoral immunity, comprising administering to the subject a therapeutic amount of CD40CR. A subject may be a non-human or, preferably, a human animal.

Immunodeficiency disorders associated with diminished humoral immunity include acquired immunodeficiency syndrome, immunodeficiency associated with malignancy or cachexia, iatrogenic immunodeficiency caused, for example, by chemotherapy or radiation therapy, as well as genetic disorders involving humoral immunity.

CD40CR may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular, or intramuscular injection, and oral, intranasal, intraocular, and rectal administration and may be comprised in micropsheres, liposomes, and/or sustained release implants.

A therapeutic amount of CD40CR for CD040 is defined as that amount which increases immunoglobulin production by at least about 30 percent.

In a further embodiment, CD40CR may be conjugated to a toxin, and then administered to a subject under circumstances in which it would be preferable to destroy B-cells that express CD40. Examples of such circumstances include patients receiving organ transplants or suffering from multiple myeloma or another B-cell malignancy, or from autoimmune disease.

CD40CR may also be used to label B-cells expressing CD40, a technique which may be useful in the diagnosis of B-cell disorders. To this end, receptor linked to an enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to B-cells in vivo or in vitro and the amount of binding may be quantitated.

CD40CR may also be used to deliver molecules that are linked to it to B-cells.

~~6. EXAMPLE A NOVEL RECEPTOR, CD40CR, ON ACTIVATED HELPER T-CELLS BINDS CD40 AND TRANSDUCES THE SIGNAL FOR COGNATE ACTIVATION OF B-CELLS~~

~~6.1. MATERIALS AND METHODS~~

~~6.1.1. ANIMALS~~

EXAMPLES

Example 1

A Novel Receptor, CD40CR, On Activated Helper T-Cells Binds CD40

And Transduces the Signal for Cognate Activation of B-Cells

MATERIALS AND METHODS

Animals

Female DBA/2J mice (Jackson Laboratories, Bar Harbor, MA) were used for the preparation of filler cells to support the growth of T_h clones and in the preparation of resting B-cells.

~~6.1.2. HELPER T-CELL CLONES (T_h)~~

Helper T-Cell Clones (T_h)

D1.6, a I-A^d-restricted, rabbit Ig-specific T_h1 clone *(Kurt-Jones et al., (1987), J Exp Med 166:1774-1787)* was obtained from Dr. David Parker, University of Mass. at Worcester. D1.6 will be referred to herein as T_h1.

~~6.1.3. ACTIVATION OF T_h BY ANTI-CD3~~

Activation of T_h by Anti-CD3

T_h1 were cultured (8 X10^6/well) in cluster wells (6 well, Corning, NY) coated with 40 μg/4 ml of PBS/well with anti-CD3 for 16 hours, as described in *(Noelle et al., (1991) J. Immunol. 146:1118-1224).*

~~6.1.4. PREPARATION OF T_h PLASMA MEMBRANES~~

Preparation of T_h Plasma Membranes

Plasma membranes were prepared by discontinuous sucrose gradient sedimentation, as described in *(Noelle et al., (1991) J. Immunol. 146:1118-1124).*

~~6.1.5 PREPARATION OF RESTING B-CELLS~~

Preparation of Resting B Cells

Resulting splenic B-cells were prepared by sedimentation on discontinuous ~~Percoll~~ PERCOLL® colloid gradients, as described in *(Defrano et al., (1982) J. Exp. Med. 155:1523)*. Cells isolated from the 70-75% (density of 1.087-1.097) ~~Percoll~~ PERCOLL® colloid gradient interface were typically >95% mIg+, had a uniform, low degree of near forward light scatter and were unresponsive to Con A.

~~6.1.6 ANTIBODIES~~

Antibodies

The following mabs were purified by ion exchange HPLC from ascites fluid of mice which had been irradiated and bone marrow reconstituted: anti-CD3:145-2C11 *(Leo et al., (1987) Proc. Natl. Acad. Sci. USA 84: 1374-1378);* anti-α, β:H57-597; anti-CD4: GK1.5 *(Wilde et al., (1983) J. Immunol. 131:2178-2183)*; anti-ICAM: YN1/1.7.4 *(Prieto et al., (1989) Eur. J. Immunol. 19:1551-1557)*; anti-LFA-1: FD441.8 *(Sarmiento et al, (1982) Immunol. Rev. 68:135)*; and anti-rat/hamster κ chain:RG-7 *(Spring, (1982) Hybrid. 1:257-273)*.

~~6.1.7. PREPARATION OF THE CD40 RECOMBINANT GLOBULIN (CD40-Ig)~~

Preparation of the CD40 Recombinant Globulin (CD40-Ig)

The CD40 fusion protein was prepared by digesting a plasmid containing a cDNA encoding the CD40 antigen *(Stamenkovic and Seed, (1989) EMBO J. 8:1403-1410)* WITH THE RESTRICTION ENZYME Pst I (P) and Sau 3A (S3). This P/S3 fragment was subcloned into the same plasmid digested with P and Bam H1 (B). This allowed the preparation of the CD40Δ was then subloned into the immunoglobulin fusion plasmid (Aruffo et al. (1990), Cell. 61:1301-1313) using a MluI and B digest. The CD40-Ig fusion protein was produced by _____ transfection in COS cells and purified on a protein A column as described in ((Aruffo et al., (1990) Cell. 61:1303-1313).

~~6.1.8. LYMPHOKINES~~

Lymphokines

Interleukin 4 (IL4): Recombinant mouse IL4 was generously provided by Drs. C. Maliszewski and K. Grabstein, Immunex Corporation, Seattle, WA.

Interleukin 5 (IL5): Recombinant mouse IL5 was purchased from R&D Research, Sarrento, CA.

~~6.1.9. INDUCTION OF B-CELL RNA SYNTHESIS BY ACTIVATED T_h PLAMSMA MEMBRANES~~

Induction of B-Cell RNA Synthesis by Activated T_h Plasma Membranes

3 X 10^4 resting B-cells were cultured in 50 μl of cRPMI in A/2 microtiter wells (Costar, Cambridge, MA). To these wells, 0.5 μg of T_h1 or T_h2 membrane protein was added. From 42-48 hrs, wells, were pulsed with 2.5 μci of ^{3}H-uridine (New England Nuclear, Boston MA), harvested, and the radioactivity determined by liquid scintillation spectroscopy. The results were expressed as cpm/culture +/-s.d.

~~6.1.10 INDUCTION OF B-CELL IMMUNOGLOBULIN SECRETION BY ACTIVATED Th PLASMA MEMBRANES AND LYMPHOKINES~~

Induction of B-Cell Immunoglobulin Secretion by Activated T_h Plasma Membranes and Lymphokines

Resting B-cells were cultured as described above. To culture wells, 0.5 μg of T_h1 membrane protein, IL4 (10 ng/ml) and IL5 (5 ng/ml) were added. On day three of culture, an additional 50 μl of Crpmi was added. On day three of culture, an additional 50 μl of cRPMI was added. On day six of culture, SN from individual wells were harvested and quantitated for IgM and IgG_1, as described in (*Noelle et al., (1991) J. Immunol.* 146*:1118-1124).*

~~6.1.11 INDUCTION OF B-CELL PROLIFERATION BY ACTIVATED T_h and IL4~~

Induction of B-Cell Proliferation by Activated T_h and IL4

$4x10^4$ resting B-cells were cultured in 50 μl of cRPMI in A/2 microtiter wells (Costar, Cambridge, MA). To these wells, $1x10^4$ resting or acctivated, irradiated (500 rads) T_h1 and Il4 (40 ng/ml) were added. On day three of cultrue, wells were pulsed with 1 μCi of ^3H thymidine, as described in *(Noelle et al., (1991) J. Immunol.* 146*:1118-1124).*

~~6.1.12 PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC TO MEMBRANE PROTEINS INDUCED ON ACTIVATED T_h1~~

Production of Monoclonal Antibodies Specific to Membrane Proteins Induced on Activated T_h1

Hamsters were immunized intraperitoneally with 5-10 x 10^6 activated T_h1 (D1.6) at weekly intervals for six weeks. When the serum titer against murine T_h1 was greater than 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NS1. SN from wells containing growing hybridomas were screened by flow cytometry on resting and activated T_h1. One particular hybridoma, which produced a mab that selectively recognized activated T_h, was further tested and subcloned to derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC.

~~6.1.13 FLOW CYTOFLUOROMETRIC ANALYSIS OF ACTIVATION MOLECULES EXPRESSED ON T_h~~

Flow Cytofluorometric Analysis of Activation Molecules Expressed on T_h

Resting and activated T_h (16 hours with anti-CD3) were harvested and incubated at 1X105 cells/50 μl with fusion protein for 20 minutes at 4°C, followed by FITC-conjugated goat anti-human (h)IgG (25 μg/ml; Southern Biotechnology, Birmingham, AL). To all samples, propidium iodide was added at final concentration of 2 μg/ml. Flow cytofluorometric analysis was performed on a BD FACSCAN. After positive gating of

cells by forward versus aide scatter, and by red negativity (for propidium iodide exclusion), the log green fluorescence of viable cells was ascertained. At least 5,000 viable cells were analyzed for the determination of percent positive cells and MFI. Staining with MR1 employed FITC-conjugated RG7, a mouse anti-rat/hamster x chain mab.

~~6.1.14 BIOSYNTHETIC LABELLING, IMMUNOPRECIPITATION, SDS-PAGE AND FLUOROGRAPHY~~

Biosynthetic Labelling, Immunoprecipitation, SDS-PAGE and Fluorography

T_h1 were rested or activated with insolubilized anti-CD3 for 16 hrs. Proteins from resting and activated T_hh1(20 x 10^6/ml) were labelled with I mCi of [^{35}S]-methionine/cysteine for one hour, at which time they were washed twice in RPMI/10%FCS and the cell pellet was lysed in extraction buffer, as described (*Noelle et al., (1991) J. Immunol.* 146*:1118-1124).* Purified antibodies or fusion proteins (1-10 μg) were added to 500ul of lysate (5x10^6 cell equivalents) at 4°C for 16 hours. At that time, the lysates were transferred to tubes containing 50 μl of packed Protein A-sepharose. The pelleted Protein A-Sepharose was resuspended and tubes were incubated at 4°C for 1 hr with agitation. The samples were then washed 3x with high stringency wash buffer. The pelleted protein A-Sepharose was resuspended in 30 μk if SDS sample buffer and run on a 10% polyacrylamide gel. After running the gel, the gel was fixed anti fluorography performed.

~~6.2.2. RESULTS~~

~~6.2.1 EFFECT OF MONOCLONAL ANTIBODIES ON THE INDUCTION OF B-CELL RNA SYNTHESIS BY PM^{Act}~~

RESULTS

Effect of Monoclonal Antibodies on the Induction of B Cell RNA Synthesis by PM^{Act}

In order to define the cell surface molecules that mediated the induction of B-cell cycle entry by PM^{Act}, mabs to T_h membrane proteins were added to cultures of PM^{Act} and B-cells. PM^{Act} induced β-cell RNA sysnthesis eight-fold over that observed with PM^{rest}) (Figure 1a). The addition of anti-LFA-1, anti-CD4, anti-ICAM-1, alone, or in combination, did not inhibit the induction of B-cell RNA synthesis by PM^{Act}.

~~6.2.2. CD40-Ig INHIBITED T-INDUCED B-CELL CYCLE ENTRY, DIFFERENTIATION AND PROLIFERATION~~

CD40-Ig Inhibited T-Induced B-Cell Cycle Entry, Differentiation and Proliferation

In the human system, it had been shown that anti-CD40 mab induced B-cell proliferation *(Clark and Lane, (1991) Ann. Rev. Immunol. 2:97-127)* thereby implicating CD40 as an important triggering molecule for B-cells. To determine if CD40 was involved in the induction of B-cell RNA sysnthesis by PM^{Act}, a soluble fusion protein of the extracellular domains of human CD40 and the F_c domain of human IgG_1 (CD40-Ig) was added to cultures of PM^{Act} derived from T_h1 and T_h2 were prepared and used to stimulate B-cell RNA sysnthesis. The addition of CD40-Ig to culture caused a dose-dependent inhibition of B-cell RNA synthesis that was induced by PM^{Act} from T_h1 and T_h2 (Fig. 1b). Half-maximal inhibition of B-cell RNA synthesis induced by PM^{Act} from T_h1 and T_h2 was about 5 μg/ml CD40-Ig. A CD7E-Ig fusion protein *(Damle and Aruffo, (1991) Proc. Natl. Acad. Sci. USA 88:6403-6407)* was without effect even when used at 25 μg/ml.

To investigate whether CD40-Ig inhibited the activation of B-cells by T-independent activators, B-cells were cultured in the presence of LPS and CD40-Ig. On

day 2, RNA synthesis was assessed (Fig. 1c). CD40-Ig was ineffective at inhibiting B-cell activation by LPS, yet inhibited the response of B-cells to PM^{Act}.

In the presence of PM^{Act}, IL4 and IL5, B-cells polyclonally differentiated to produce Ig *(Hodgkin et al., (1990) J. Immunol. 145:2025-2034; (Noelle et al., (1991) J. Immunol. 146:1118-1124)*. To evaluate the requirements for CD40 signalling in this process, CD40-Ig was added at the initiation of culture, or on subsequent days of culture. The addition of CD40-Ig (Fig. 2a) at the initiation of culture inhibited greater than 95% of polyclonal IgM and IgG_1 production compared to control levels in its absence. In contrast, the addition of CD40-Ig on day 1 and 2 of culture showed little, if any, inhibitory effect on IgM and IgG_1 production. These data indicated that after 24 hours, signalling via CD40 is no longer essential for the differentiation of B-cells to Ig secretion.

Data thus far indicated that CD40 was implicated in the activation of B-cells by PM^{Act}. Studies were performed in order to ensure that CD40 was also involved in the activation of B-cells by intact, viable, activated T_h. T_h1 were activated for 16 hours with insolubilized anti-CD3, harvested and irradiated. The irradiated T_h1 were cultured with B-cells in the presence of IL4 and B-cell proliferation was determined on day 3 of culture. An exogenous source of IL4 was required to achieve B-cell proliferation with T_h1, because T_h1 do not produce IL4 *(Noelle et al., (1989) J. Immunol. 143:1807-1814)*. CD40-Ig inhibited the induction of B-cell proliferation by irradiated T_h in a dose-dependent manner, similar to that observed with PM^{Act} (Fig. 2b). The Megative control, CD7E-Ig, exerted no appreciable effect.

~~6.2.3 CD40-Ig DETECTED A MOLECULE EXPRESSED ON ACTIVATED, BUT NOT RESTING T_h~~

CD40-Ig Detected a Molecule Expressed on Activated, but not Resting T_h

To investigate whether activated T_h1 express a binding protein for CD40, resting and activated (1d6 hours) T_h1 were stained with CD40-Ig or CD7E-Ig, followed by FITC-anti-HigG. Binding of CD40-Ig was assessed by flow cytometry (Fig. 3). T_h1 that were activated for 16 hours with anti-CD3, but not resting T_h1, stained 56% positive with CD40-Ig, but not with the control CD7E-Ig. To identify the CD40-Ig binding protein, T_h1 proteins were biosynthetically labelled with [^{35}S]-methionine/cysteine and proteins immunoprecipitated with CD40-Ig or CD7E-Ig. The immunoprecipitated proteins were resolved by SDS-PAGE and fluorography (Figure 4). A prominent band with an apparent molecular weight of 3g kD immunoprecipitated in a dose-dependent manner with 1 and 10 μg of CD40/sample. As controls, anti-class I mab immunoprecipitated bands at 55 kD and a low molecular weight band, β2 microglobulin. In the absence of mab, no prominent bands were visible. A 39 kd band was also immunoprecipitated from activated T_h that were vectorially labelled with ^{125}I, confirming that the 39kD protein was a membrane protein.

~~6.2.4 MONOCLONAL ANTIBODY MR1, SPECIFIC TO 39Kd Th MEMBRANE PROTEIN, INHIBITED THE INDUCTION OF B-CELL RNA SYNTHESIS BY PM^{Act}~~

Monoclonal Antibody MR1, Specific to 39Kd Th Membrane Protein Inhibited the Induction of B-Cell RNA Synthesis by PM^{Act}

Mabs specific to antigens selectively expressed on activated versus resting T_h were developed to identify T_h molecule(s) responsible for the T_h effector phase activity. One such mab, MR1, recognized an antigen that was selectively expressed on activated T_h1. To investigate whether MR1 and CD40-Ig recognized the same molecule, flow cytometry and blocking studies were performed. CD40-Ig and MR1 stained approximately 56% and 61%, respectively, of activated, but not resting Th (Fig. 5a). MR1, but not another hamster anti-T cell mab, anti-α/β TCR, blocked the staining of

activated T_h1 with CD40-Ig, in a dose-dependent manner. These data suggested that CD40-Ig and MR1 recognized oerlapping or identical eptiopes on the 39 kD Th protein. To further demonstrate that CD40-Ig and MRI recognized the same molecule, the antigen that bound MR1 was identified by immunoprecipitation of proteins from radiolabelled Th lysates. Both CD40-Ig and MR1 immunoprecipitated a 39 kD protein (Fig. 5b). Finally, immunoprecipitation of the 39kD protein with CD40-Ig removed the antigen recognized by MR1 from radiolabelled lysates of activated T_h supporting the tenet that MR1 antigen and the CD40 binding protein were identical.

Functional studies were performed with MR1 to address whether theis mab neutralized the activity expressed by PM^{Act}. PM^{Act} and B-cells were cultured alone, or in the presence of hamster mabs or CD40-Ig. Two hamster mabs, anti-α/β TCR and α-CD3 did not inhibit the activation of resting B-cells by PM^{Act}. In contrast, MR1 or CD40-Ig inhibited B-cell activation (Fig. 6).

~~6.3 DISCUSSION~~

SUMMARY

The data show that blocking of prominent T_h surface molecules (LFA-1, CD4, ICAM-1, CD3, α, β TCR) with mabs did not impede the capacity of activated T_h to induce B-cell cycle entry. In contrast, CD40-Ig or a maB specific to the CD40 binding protein, blocked T_h-dependent B-cell activation in a dose-dependent manner. Furthermore, the CD40 binding protein was identified as a 39 kD protein that is selectively expressed on the membranes of activated, but not resting T_h. Both CD40-Ig and a mab specific to the 39kD CD40 binding protein blocked B-cell activation by PM^{Act}.

Although a number of membrane proteins have been implicated in T_h-dependent B-cell signalling, evidence presented herein dismisses the contribution of some molecules

(LFA-1, CD4, CD3, α, β TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate signalling by T_h. Data show that CD40-Ig and a mab specific to the CD40 binding protein inhibits T_h-dependent B-cell activation.

The ligand for CD40 is a 39KD protein that is expressed on activated, but not resting T_h. Biochemical studies indicate that the 39kD protein is a single chain molecule since electrophoretic migration was not influenced by reducing agents. Based on the functional studies presented in this study, both activated T_h1 and T_h2 express the 39 kD CD40 binding protein. This is consistent with the functional studies that show both T_h1 and T_h2 induce B-cell cycle entry. In an attempt to further characterize the 39 kD protein, cDNA encoding CD proteins in the MW range of 39kD (CD 53, CD27 and CD69) were transiently transfected into COS cells and the cells were tested for CD40-Ig binding. None of the transfected COS cells expressed proteins that bound CD40-Ig. It is therefore suspected that the 39 kD protein is not one of these CD proteins.

The biochemical basis for signal transduction between T_h and B-cells has been elusive. The identification of CD40 as the signal transducing molecule for T cell help focusses attention on specific biochemical pathways known to be coupled to the CD40 molecule. CD40 is a member of the nerve growth factor receptor (NGFR) family by the virtue of the presence of four cysteine-rich motifs in its extracellular region. Signaling through CD40 by mab has been shown *(Uckun et al., (1991) J. Biol. Chem. 266:17478-17485)* to involve the activation of tyrosine kinases resulting in the increased production of inositol trisphosphate and the activation of at least four distinct serine/threonine kinases. Based on information obtained from signaling through other members of the NGF receptor family, it is anticipated that interaction between activated T_h and B will result in many of the same biochemical processes.

~~7. EXAMPLE: BINDING OF CD40 Ig TO HUMAN T-CELL LINES~~

Example 2

Binding of CD40 Ig to Human T-Cell Lines

For immunofluorescence binding studies, CD40 Ig fusion protein was conjugated with biotin using biotin-succinimide (Sigma). Flow cytometry analysis was then performed by tow-step staining using phycoerythrin (PE)-strepavidin (Bectin-Dickinson) with a Coulter Epics C instrument. Representative results of screening multiple T cell lines is presented below. The Jurkat and HSB2 cell lines were found to bind specifically, whereas other T cell lines including CEM, HPBALL, and murine thymoma did not bind the CD40 Ig fusion protein (Fig. 7).

Various publications are cited herein which are hereby incorporated by reference in their entirety.

The hybridoma identified in this application as MR1 was deposited on May 22, 1992 with the American Type Culture Collection, ATCC, International Depository Authority, ~~12301 Parklawn Drive, Rockville, Maryland 20852~~ 10801 University Boulevard, Manassas, VA 20110-2209, in compliance with the Budapest Treaty, and accorded Accession Number ATCC HB 11048. All restrictions as to the availability to the public of the hybridoma cell line MR1 will be irrevocably withdrawn upon issuance of a United States Patent to this application. Also, access to the MR1 cell line will be available to the Commissioner during the pendency of this patent application or to one determined by the Commissioner to be entitled to such cell line under 37 C.F.R. §1.14 and 35 U.S.C. §122.